# PHYSIOLOGICAL AND MOLECULAR MECHANISMS UNDERLYING THE INTEGRATION OF LIGHT AND TEMPERATURE CUES IN ARABIDOPSIS THALIANA SEEDS

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**A brief summary statement (3-4 sentences maximum):**

This work studies the function of alternating temperatures in dormancy and germination of *Arabidopsis thaliana* seeds. We demonstrated that the incubation of dormant seeds at 15°C/23°C alternating temperature cycles for two days is sufficient to enhance the light response of seeds. The results suggest a functional role for some components of the circadian clock related with the action of DOG1 for the integration of alternating temperatures and light signals in the relief of seed dormancy.

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**SUMMARY**

The relief of dormancy and the promotion of seed germination are of extreme importance for a successful seedling establishment. Although alternating temperatures and light are signals promoting the relief of seed dormancy, the underlying mechanisms of their interaction in seeds are scarcely known. By exposing imbibed *Arabidopsis thaliana* dormant seeds to two-day temperature cycles previous of a red light pulse, we demonstrate that the germination mediated by phytochrome B requires the presence of functional *PRR7* (*PSEUDO-RESPONSE REGULATOR 7*) and *TOC1* (*TIMING OF CAB EXPRESSION 1*) alleles. In addition, daily cycles of alternating temperatures in darkness reduce the protein levels of DOG1, allowing the expression of *TOC1* to induce seed germination. Our results suggest a functional role for some components of the circadian clock related with the action of DOG1 for the integration of alternating temperatures and light signals in the relief of seed dormancy. The synchronization of germination by the synergic action of light and temperature through the activity of circadian clock might have ecological and adaptive consequences.

**Keywords:** Seed dormancy, alternating temperature, light, circadian clock, DOG1, Arabidopsis.

**INTRODUCTION**

Seed dormancy plays a central role in the adjustment of plant populations to their environment. Processes that modulate dormancy depth and its alleviation define the germination timing of seed population and are consequently of the outmost adaptive importance (Donohue *et al*. 2005). During their development in the mother plant, seeds of most plant species enter in a state of dormancy. After seeds fall to the ground, they can show cyclic changes in sensitivity to environmental signals during the time of burial (Bouwmeester *et al*. 1994; Derkx & Karssen, 1994). A well-known genetic block of germination is DOG1 (*DELAY OF GERMINATION 1*), whose protein accumulates during seed maturation and its abundance in freshly harvested seeds determines their dormancy level (Graeber *et al.* 2014). Reduced dormancy levels in seeds were associated with the inactivation of DOG1 protein, and its expression fluctuates during the year in buried seeds of *Arabidopsis thaliana* accompanying variations in cyclic dormancy (Footit *et al.* 2013). The expression of Abscisic Acid (ABA) - and Gibberellins (GA)-related genes also change in harmony with those variations (Footitt *et al*. 2011).

When the dormancy level is low enough, germination can be induced by environmental cues that predict favourable conditions for the development of the new plant. The three main factors that can terminate dormancy are light, alternating temperatures and nitrates (Benech-Arnold *et al*. 2002). Light is the best known of the three, and therefore there is a need to advance filling the gaps in the information about the others. Particularly, alternating temperatures have been shown to be, in many species, an effective signal providing the seeds information about the depth they are buried in the soil and the extent of soil covering (Benech-Arnold *et al*. 1988; Ghersa *et al*. 1992). Although there are species that only need alternating temperatures to exit from dormancy and initiate germination, there is a large

number of species demanding both alternating temperatures and light (Baskin & Baskin, 1998).

Red (R) and Far-Red (FR) light are perceived by the phytochrome photoreceptor system. Phytochromes are synthesized in the inactive form, Pr (maximum absorption in R) and are transformed by light into the active form, Pfr (maximum absorption in FR). The Arabidopsis genome encodes five phytochromes (phyA to phyE). phyB has a prominent role as the photoreceptor regulating the R/FR reversible response for germination (Shinomura *et al*. 1994; Botto *et al*. 1995), and phyE contributes to this regulation in *phyA phyB* double mutant seeds (Henning *et al*. 2002), indicating redundancy in phytochrome functions in the R-mediated control of seed germination. In some conditions, phyA is the main photoreceptor of germination promotion by FR light (Botto *et al*. 1996; Shinomura *et al*. 1996), and phyE and phyD can contribute to this response in the absence of phyA (Henning *et al*. 2002; Arana *et al*. 2014). It is well documented that seeds in soil banks can acquire high (i.e., R/FR reversible response) and very high (i.e., FR-responsive seeds) light sensitivity according to the extent of after-ripening and burial conditions (Scopel *et al*. 1991; Botto *et al*. 1998).

The interaction between phytochromes (Pfr) and PIF1 initiates a vast alteration in the pattern of gene expression in different tissues of the seed, directly or indirectly influencing the metabolism or sensitivity of several hormones and cell wall proteins (Oh *et al*. 2004, 2007). The promotion of germination depends on changes in the levels of hormones, mainly those of GA and ABA. Alleviation of physiological dormancy and the induction of germination requires high GA:ABA ratio; accordingly, the reduction in PIF1 levels produces large changes in gene expression leading to an increase in GA:ABA ratio. In spite of the importance of alternating temperatures on the relief of seed dormancy, there is scanty

information on the action of this signal cue on ABA and GA-related gene expression. Alternating temperatures increase the GA:ABA ratio in *Cynara cardunculus* seeds by decreasing the content and sensitivity to ABA and involving a reduction in the expression of *NCED (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE*) and *ABI5 (ABA INSENSITIVE 5)* (Huarte *et al*. 2014). Correspondingly, in a transcriptome study with *Euphorbia esula* seeds, a decrease in the expression of ABA-related genes, including *ABI5*, are induced by alternating temperatures (Foley *et al*. 2010). In other cases, alternating temperatures are necessary for the phytochrome promotion of GA metabolism that increases the expression of *GA3ox1 (GIBBERELLIN 3-OXIDASE 1)* (Arana *et al*. 2007).

Some of the environmental signals that regulate plant growth and development are strongly related to components of the circadian clock. It is well known that the circadian clock can be regulated by light, temperature, and nitrate (Gutierrez *et al*. 2008; Shim *et al*. 2017). Moreover, the functioning of the clock also alters hormone metabolism (Imaizumi, 2010). Consequently, the circadian clock might be involved in the control of dormancy in seeds. In fact, the clock genes *CCA1 (CIRCADIAN CLOCK ASSOCIATED 1), LHY (LATE ELONGATED HYPOCOTYL), GI (GIGANTEA)* and *TOC1 (TIMING OF CAB EXPRESSION 1)* have been proposed to be involved in the relief of dormancy by different environmental signals in Arabidopsis seeds (Penfield & Hall, 2009). In this study, we extend the knowledge of the involvement of clock genes in the control of germination showing their association with alternating temperatures previously exposing the seeds to a light pulse.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*A. thaliana* plants were grown under white light with PAR = 100 µmol.m$^{-2}$.s$^{-1}$ and an average temperature of 21°C ± 2°C. The plants were grown together and their mature seeds were harvested at the same time. Seeds of each genotype were collected as a single bulk consisting of at least 10 plants. In all the experiments, we used seeds recently harvested (0-30 d-old after-ripened seeds). We stored the seeds in darkness into opened tubes contained in a box with silica gel at 4°C to maintain the status of primary dormancy until use them in the experiments. We used seeds of Col-0, L*er* and CVI accessions and NILDOG17-1 carrying the strong allele of CVI-DOG1 in L*er* background (Bentsink *et al*. 2006), *phyA-211* (Salk_014575C), *phyB-9* (Salk_069700), *phyA-211 phyB-9* (Reed *et al*., 1994), *dog1* (Salk_000867), DOG1-OX (a gift from Leónie Bentsink), *prr7-3* (Salk_030430), *toc1-1* (CS 3756), *cca1-1* and *cca1-1 lhy-21* (a gift from Rachel Green), and *ProDOG1*:*3xHA*:*DOG1* (hereafter named as DOG1-HA, Nakabayashi *et al*. 2012).

### Germination conditions, light and temperature treatments, and statistical analyses

In order to measure the influence of different pre-incubation temperature treatments on germination responsiveness to light, seeds were sown in petri dishes containing 25 mL of 0.8% (w/v) agar in demineralized water. After one hour of imbibition at ambient temperature, seeds were treated with a saturated FR pulse (FRp, calculated Pfr/P= 0.03, 42 µmol.m$^{-2}$.s$^{-1}$) to minimize the quantities of active phytochrome formed during their development in the mother plant. Then, the petri dishes with seeds were wrapped with black plastic bags, and incubated at 15°C/23°C, 16h/8h temperature cycles for two days in darkness. We included three additional daily constant temperatures at 15°C, 17.5°C and

23°C as control of minimum, average and maximum temperatures of the alternating daily temperature regime. Since 17.5°C and 15°C/23°C pre-incubation temperature treatments provided a similar accumulation of thermal time, we discarded effects of differential thermal time on seed germination. After the pre-incubation temperature treatments, the seeds were irradiated with a saturated R pulse (Rp, calculated Pfr/P= 0.87, 0.05 µmol.$m^{-2}$.$s^{-1}$) or a saturated FRp for 3 min before the incubation of them at 17.5°C in darkness for 5 days until germination was recorded.

Each replicate consisted of 20 seeds per genotype and temperature/light treatment. The average response in each condition was evaluated in at least three biological replicates. Four independent experiments with different seed batches were conducted. Statistical analyses were performed using two-way ANOVA with temperature (T) and genotype (G) as factors. For statistical analyses, the percentage of germination was transformed using angular transformation, and percentage of germination was plotted using GraphPad Prism Software version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). When treatment-by-genotype (T x G) interaction was significant at $P < 0.05$, Bonferroni post-test was used to test mean differences.

**Gene expression analysis by quantitative RT–PCR**

For gene expression analysis, each replicate consisted of 5-10 mg of seeds incubated for two days in darkness at 15ºC/23°C or 17.5°C. Seed samples were harvested at ZT (zeit-geber time)= 0, 4, 8, 12, 16, 20 or 24 hours. We named as ZT to the period of seed incubation in darkness at 17.5°C after the end of the pre-incubation temperature treatment. After sampling, seeds were immediately frozen in liquid nitrogen and stored at -80°C. RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Steinheim, Germany)

according to manufactures protocol. cDNA was synthesized using MMLV High Performance Reverse Transcriptase (Epicentre, Madison, USA) and oligo-dT primers. The synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche, Madison, USA) using the 7500 Real Time PCR System cycler (Applied Biosystems, Foster City, CA, USA). *PP2A* gene was used as a normalization control (Czechowski *et al*. 2005). The primers used are described in Supplemental Table 1.

**Quantification of protein levels**

Seeds were sown and pre-incubated in darkness at 15°C/23°C or 17.5°C for two days and harvested at ZT= 0. We also harvested the material for immunoblot analysis at one hour after sowing as a control of DOG1 protein level before the pre-incubation treatments. Total proteins of three biological replicates (10 mg of seeds) were extracted using plant protein extraction buffer [50 mM Tris-HCl 50 pH=7.5, 150mM NaCl, 10% Glycerol, 10 mM EDTA pH=8, 0.1% Sarcosyl, 10 mM □-mercaptoethanol and 1 mM PMSF (Protease Inhibitor Cocktail Tablets, Roche, Madison, USA)]. Protein content was quantified by the Bradford assay (Bradford, 1976). Equal amounts of protein were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting analysis. DOG1 immunodetection was performed using anti-HA (Anti-HA 12CA5, Roche, Madison, USA). Goat anti-mouse immunoglobulin G horseradish peroxidase (IgG HRP) conjugate secondary antibody (Invitrogen, Madison, USA) was used for detection. We used DOG1-HA transgenic line and Col-0 as a negative control (data not shown). The results are expressed as the ratio of DOG1-HA to IgG and normalized to the one-hour-imbibition treatment. For statistical analysis, we proceeded as commented in the previous section, with significant differences taken at P values ≤ 0.1.

## RESULTS

### Daily temperature cycles promote phytochrome B-mediated germination

Wild-Type Col-0 seeds were incubated in darkness at temperature cycles (15°C/23°C) or constant temperatures (15°C, 17.5°C, 23°C) for two days prior to irradiation with a red light pulse (Rp) or a far-red light pulse (FRp). Germination was counted after 5 days at 17.5°C in darkness (Fig. 1). The Rp light treatment induced 81% of germination in seeds exposed to 15°C/23°C daily temperature cycles, whereas the germination was significantly lower for seeds incubated at constant temperatures: 51%, 40% and 10% at 15°C, 17.5°C and 23°C, respectively (Fig. 1b). No significant differences between seed germination at alternating temperatures vs. constant temperatures were observed when the seeds were exposed to a FRp or in darkness (Fig. 1b). Therefore, we conclude that, in our experimental conditions, daily temperature cycles promote R-mediated seed germination in Arabidopsis seeds. Since germination induced by a Rp can be mediated by different phytochromes (Botto *et al*. 1996; Henning *et al*. 2003; Arana *et al*. 2014), we analyzed the behaviour of *phyA, phyB* and *phyA phyB* mutant seeds in our temperature conditions before the irradiation with a Rp (Fig. 1c). Col-0 and *phyA* seeds germinated close to 80% at daily temperature cycles and less than 50% at constant temperatures, while *phyB* and *phyA phyB* seeds germinated less than 20%, independently of the temperature treatment (Fig. 1c). These results demonstrate that daily temperature cycles promote the phyB-mediated germination in Arabidopsis seeds.

### Daily temperature cycles activate the expression of genes involved in ABA and GA metabolism and cell wall growth

We studied the expression of some genes known to be associated with seed germination. We evaluated gene expression related with GA (*GID1A*, *GA3ox1* and *GA20ox3*), ABA (*NCED9*

and *CYP707A2*) and cell wall growth (*EXP10*). RNA was extracted from Col-0 seeds incubated for two days under 17.5°C or 15°C/23°C in darkness. Immediately after the pre-incubation temperature treatment, we harvested the material to perform the qRT-PCRs. Gene expression increased in those genes associated with the promotion of germination at 15°C/23°C daily temperature cycles compared with those imbibed at 17.5°C constant temperature (Fig. 2). Daily temperature cycles promoted the expression of GA signalling or metabolism associated genes (*GID1A, GA3ox1* and *GA20ox3*) and one ABA catabolic gene (*CYP707A2*) together with a dramatic increase in the expression of *EXP10* (more than 130 fold, Fig. 2). In agreement, *NCED9* expression, an anabolic ABA gene, was significantly reduced at 15°C/23°C compared with 17.5°C (Fig. 2). These results demonstrate that daily temperature cycles promote seed germination through changes in expression of GA and ABA metabolic genes, as well as those genes necessary for cell wall softening before radicle protrusion.

**_PRR7_ and _TOC1_ functional alleles promote seed germination in response to daily temperature cycles**

It has been shown that daily temperature cycles affect the performance of the circadian clock (Harmer *et al*. 2009). In order to have a better understanding of the functional roles played by some circadian clock components in seed germination induced by a Rp after daily alternating temperature cycles, we studied the behaviour of different clock mutants under our experimental conditions (Fig. 3). Wild type Col-0 seeds germinated more than 70% at 15°C/23°C daily temperature cycles, and fewer than 40% at 15°C, 17.5°C or 23°C constant temperatures. *cca1-1* and *cca1-1 lhy-21* mutant seeds showed a similar germination pattern than Col-0 seeds, suggesting that neither *CCA1* nor *LHY* are required for the promotion of R-

mediated seed germination by alternating temperatures (Fig. 3a). Interestingly, *prr7-3* and *toc1-1* mutant seeds did not respond to 15°C/23°C showing a similar percentage of germination to those incubated at 15°C or 17.5°C. These results demonstrate that the promotion of R-mediated seed germination by daily temperature cycles requires functional alleles of *PRR7* and *TOC1* (Fig. 3a).

Taking into account the promoting function of TOC1 and PRR7 on seed germination induced by daily temperature cycles, we asked whether the expression of both genes is altered by the pre-incubation temperature condition before exposing the seeds to a Rp light. We evaluated gene expression in Col-0, *prr7-3* and *toc1-1* seeds. RNA samples were extracted between ZT=0 and ZT=24, every four hours, after the pre-incubation treatments at 15°C/23°C or 17.5°C in darkness (Fig. 3b). Daily temperature cycles promoted *TOC1* expression in Col-0 seeds with a peak of expression between ZT=8 and ZT=12, and some hours later for *PRR7* (ZT=16). In both cases, the lowest expression was during the night at ZT=20. These patterns of gene expression were not observed when the seeds were incubated at constant 17.5°C. Consistent with these results, *prr7* and *toc1* mutant seeds showed the same null pattern of *TOC1* and *PRR7* expression, respectively, at 15°C/23°C or 17.5°C (Fig. 3b). These results demonstrate that the promotion of phyB-mediated germination by daily temperature cycles requires the presence of functional alleles of *PRR7* and *TOC1*, whose cyclic pattern of gene expression is stimulated in response to alternating temperatures in darkness.

**DOG1 negatively regulates seed germination promoted by daily temperature cycles**

Taking into account that *DOG1* is a major dormancy gene that inhibits seed germination in Arabidopsis and other species (Bentsink *et al*. 2006; Graeber *et al.* 2014), we evaluated the

role of DOG1 in the response to daily temperature cycles. We studied the germination of Col-0 and *dog1* seeds at 17.5°C and 15°C/23°C (Fig. 4). In Col-0 seeds, daily temperature cycles induced 85% of germination, whereas only 26% of seeds geminated after the incubation of them at 17.5°C (Fig. 4a). Interestingly, *dog1* seeds germinated over 80% independently of the pre-incubation temperature condition (Fig. 4a). These results suggest that DOG1 inhibits the germination at 17.5°C. We next asked if *TOC1* and *PRR7* expression change differentially under constant or daily temperature cycles in the absence of DOG1 before the exposure of seeds to a Rp. Daily alternating temperatures promoted *TOC1* expression in Col-0, but not in *dog1* seeds. Interestingly, *dog1* seeds showed a similar and high accumulation of *TOC1* transcripts at constant and alternating temperature cycles (Fig. 4b), suggesting that DOG1 inhibits seed germination under constant temperatures by repressing *TOC1* expression. The *PRR7* expression at ZT=0 did not change by daily temperature cycles in Col-0 seeds, probably because its peak of expression is at ZT=16 (Fig. 3b). The *PRR7* expression was reduced independently of the pre-incubation temperature condition in *dog1* seeds (Supporting Information Fig. S1). These results demonstrate that *DOG1* inhibits seed germination under constant temperature, in part, by the repression of *TOC1* expression. Furthermore, the overexpression of *DOG1* in Col-0 background and the expression of strong alleles of DOG1-CVI accession in L*er* background reduced the effects of alternating temperatures in the promotion of R-mediated seed germination (Supporting Information Fig. S2), confirming the relevance of *DOG1* mediating alternating temperature response in different genetic backgrounds.

Furthermore, we studied DOG1 protein activity in DOG1-HA seeds incubated at alternating and constant pre-incubation temperatures. We extracted total proteins at ZT=0 in DOG1-HA seeds imbibed in darkness at 15°C/23°C and 17.5°C for two days. Protein levels were

normalized to DOG1 level in seeds imbibed in darkness for one hour after sowing. Daily temperature cycles significantly reduced DOG1-HA protein levels compared to those seeds incubated at 17.5°C (Fig. 5), suggesting that alternating temperatures reduce dormancy through changes in the expression of DOG1.

**DISCUSSION**

Alternating temperatures cycles are an environmental signal that relief seed dormancy and, consequently, contribute to define the dynamics of emergence of weed seedlings in the field (Benech-Arnold *et al*. 2000). Unfortunately, this process has not been extensively studied in Arabidopsis-model-seed system with recent exceptions (Footit *et al*. 2017; Tophan *et al*. 2017). By mathematical and experimental approaches, Bassel´s team demonstrated that alternating temperatures act as an instructive signal in the root tip of Arabidopsis dormant seeds that define the distribution of hormone metabolites and ABA/GA transporter activity to break dormancy (Tophan *et al*. 2017). Here we demonstrate that the incubation of dormant seeds at 15°C/23°C alternating temperature cycles for two days is sufficient to enhance the light response of seeds. Interestingly, the pre-incubation of seeds at 17.5°C, that establishes identical accumulative thermal time than daily temperatures cycles, significantly and consistently showed lower levels of germination (Fig. 1b), indicating a synergism between alternating temperatures and light signals to end dormancy. Although alternating temperatures in the dark enhance the expression of some of the genes frequently associated with the promotion of germination, that promotion alone is obviously not enough to induce germination, because seeds exposed to a FRp did not show differences on germination between alternating vs. constant temperatures (Fig.

1b). Seeds of other accessions with shallower primary dormancy than Col-0, like L*er* accession, or with stronger primary dormancy, like CVI accession also display responses to alternating temperatures although the magnitude and stability of the response is variable (Supporting Information Fig. S2).

The response to alternating temperatures followed by light is mediated exclusively by phyB, since *phyB* mutant seeds did not respond to these signals (Fig. 1c). However, we should not rule out that other stable phytochromes or the unstable phyA can be responsive to alternating temperatures if the light sensitivity of seeds changes during after-ripening or burial conditions. For example, vegetation cover, depth of seed burial, soil moisture, and after-ripening can change the light sensitivity of weed seeds depending on previous burial conditions (Botto *et al*. 1998; Botto *et al*. 2000; Battla *et al*. 2007). In addition, although phyA and phyB are the principal photoreceptors responsible for FR- and R-induced germination, respectively (Botto *et al*. 1995; Botto *et al*. 1996; Shinomura *et al*. 1996), other phytochromes can promote germination in the absence of phyB and phyA (Henning *et al*. 2002; Arana *et al*. 2014). It is also interesting to note that the ability of a FR pulse to promote germination also varies over a 24-hour period, suggesting a circadian input into the germination program (Oliverio *et al*. 2007). All these evidences suggest that, although phyB is responsible for the synergism between alternating temperatures and light in our experimental conditions, other phytochromes might replace its function in other environmental contexts. In seedlings, phyB participates in temperature perception; its thermal reversion rate from the active Pfr state to the inactive Pr form increases in the range between 4°C and 30°C, allowing cell expansion and hypocotyl growth (Jung *et al.* 2016; Legris *et al.* 2016). Interestingly, phyB mutants still keep the thermal-dependent growth response (Mazzella *et al.* 2000; Halliday & Whitelam, 2003), revealing the existence

of alternative thermosensory pathways different to phyB. We irradiated the seeds with a saturating FRp immediately after imbibition; therefore, the quantities of Pfr of the stable phytochromes are minimum when seeds are exposed to the different thermal regimes in darkness. phyA showed no detectable reversion in Columbia and Landsberg ecotypes (Eichenberg *et al.* 2000). Therefore, in our experimental conditions, other temperature sensing mechanisms not necessary involved in light perception, i.e. those involving chromatin remodelation (Huff & Zilberman, 2012), might participate in the responses of the seeds to the temperature.

The expression of GA, ABA and cell growth genes promoting germination was activated in seeds imbibed at 15°C/23°C, but not when they were incubated at 17.5°C constant temperature (Fig. 2). The promotion of germination by alternating temperatures required the expression of the clock genes *TOC1* and *PRR7* (Fig. 3). Interestingly, other circadian clock genes do not seem to be necessary for the promotion of germination in our experimental conditions, since *lhy-21* and *cca1-1* mutant seeds responded normally to alternating temperatures (Fig. 3). *TOC1* expression is repressed by LHY and CCA1 proteins binding directly to its promoter around dawn (Alabadí *et al*. 2001; Mizoguchi *et al*. 2002). In addition, TOC1 protein increased in the evening and activates *CCA1* expression partially by antagonizing the action of CHE, a transcriptional repressor of *CCA1* (Pruneda-Paz *et al*. 2009). Other *PRR* genes belonging to the morning loop are required for the proper timekeeping of circadian clock (Matsushika *et al*. 2000). The fact that the induction of germination by daily temperature cycles requires the expression of specific clock components (Fig. 3) strongly suggest that, in our experimental conditions, the control of germination by temperature involves the regulation of a sub-set of clock genes, which are promoted in specific temporal windows during the day. In other words, shortly after

imbibition, the daily temperature cycles might affect specific clock components that are linked to the regulation of dormancy and germination, rather than the overall performance of the circadian clock. In a dormancy cycling simulated experiment using clock mutants, Footit et al. (2017) found that clock elements belonging to the central loop (i.e., CCA1, LHY and TOC1) contribute to reduce the ABA sensitivity, and mutant seeds in these genes showed a faster entrance in secondary dormancy at 25°C. In contrast, components of the clock belonging to the early loop (i.e., PRR5, PRR7 and PRR9) increased ABA sensitivity, and mutant seeds in PRR genes had a lower entrance in secondary dormancy. In addition, a previous work found that the germination of *cca1 lhy* double mutant seeds exposed to dark/light cycles and 17°C/27°C alternating temperatures was higher than the WT, whereas *CCA1* and *LHY* overexpressing lines showed an opposite phenotype suggesting that both clock components can inhibit seed germination in this condition (Penfield & Hall, 2009). It has also been demonstrated that *CCA1* and *GI* clock genes can regulate seed germination in response to stratification and post-maturation signals (Penfield & Hall, 2009), and *CCA1* but not *LHY* expression is involved in the response of seedlings to nitrate (Gutierrez *et al*. 2008). All these evidences suggest that different clock genes might integrate environmental input signals to adjust a specific developmental program such as the relief or induction of dormancy, the promotion of seed germination or the growth of seedlings.

Here, we demonstrate that *DOG1*, a well-known dormancy gene, inhibited the response to diurnal cycles of alternating temperatures by repressing germination and *TOC1* expression at constant temperatures in Arabidopsis seeds (Fig. 4). In addition, alternating temperatures significantly reduced DOG1 protein levels (Fig. 5) and this might allow the expression of *TOC1* gene. In agreement with these results, the overexpression of *DOG1* inhibited the promotion of R-mediated seed germination by alternating temperatures (Supporting

Information Fig. S2). Furthermore, the influence of alternating temperatures was variable between accessions with different levels of dormancy: no response for CVI, a genotype with high levels of dormancy, and intermediate and high responses for L*er* and Col-0, respectively. Correspondingly, strong alleles of *DOG1* from CVI reduced the response to alternating temperatures in the NILDOG17-1 seeds (Supporting Information Fig. S2). Our results did not discriminate whether *DOG1* alters the response to alternating temperatures in a direct or indirect manner. However, we found that TOC1 promoted seed germination in response to alternating temperature cycles (Fig. 3) and DOG1 repressed *TOC1* expression at constant temperature (Fig. 4). We speculate that the reduction of DOG1 protein levels by alternating temperature cycles allows the daily patterns of *TOC1* expression in darkness. Interestingly, DOG1 inhibits the expression of GA biosynthesis and endosperm weakening genes in a temperature dependent manner to control the timing of germination (Graeber *et al*. 2014). In fact, the overexpression of DOG1 in *Arabidopsis thaliana* and *Lepidium sativum* seeds delayed the germination response at constant temperature (24°C) compared to a cooler temperature (18°C) by altering the GA biosynthesis catalyzed by *GA20ox* and impeding the expression of endosperm weakening genes (Graeber *et al*. 2014).

We conclude that circadian clock elements are involved in the integration of alternating temperatures and light signals in the presence of active dormancy alleles for the fine adjustment of dormancy relief. The synchronization of the germination process by the synergic action of both environmental signals might have ecological and adaptive consequences for the persistent of species in seed banks.

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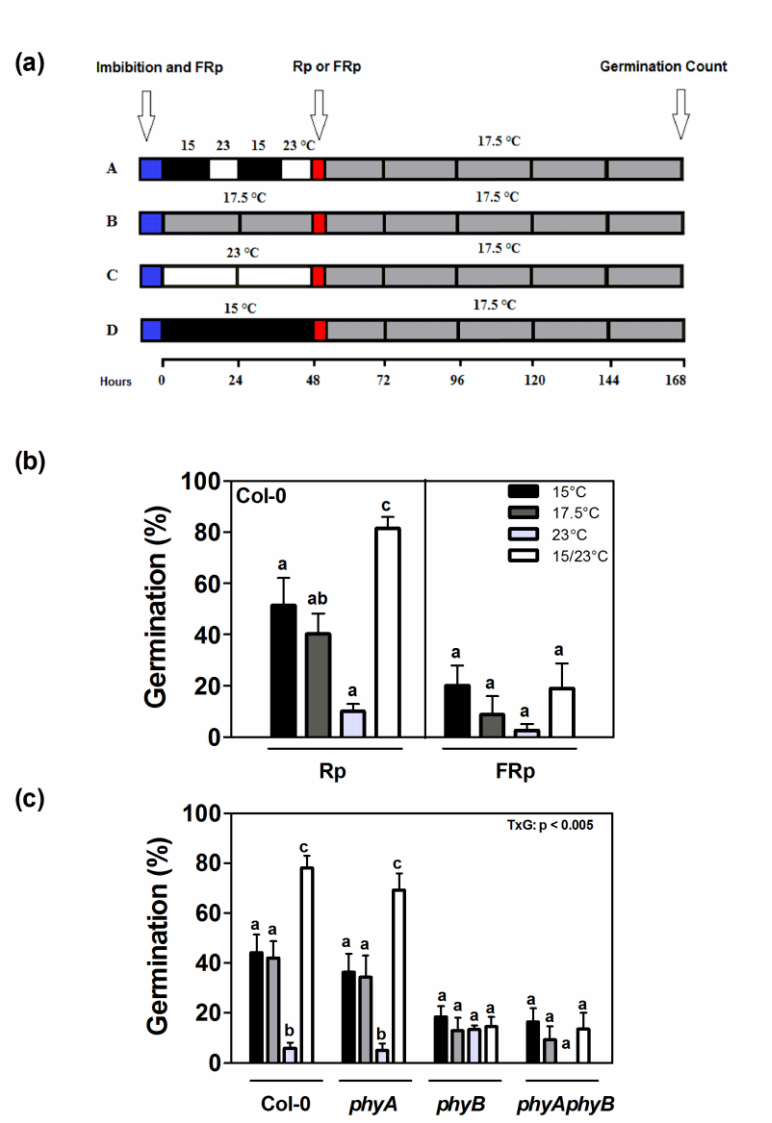


**Figure 1. Daily temperature cycles promote R-mediated seed germination through the action of phyB.** (a) Protocol used for the experiments: seeds were imbibed for one hour, irradiated with a far red pulse (FRp) and incubated in darkness at daily temperature cycles (15°C 16h/23°C 8h) or at the constant control temperatures of 15°C, 17.5°C or 23°C for 48 hours. The seeds were then irradiated with a red pulse (Rp), FRp or kept in darkness. Seeds were then incubated in darkness at constant temperature (17.5°C) for five days until germination was counted. (b) Germination of Col-0 seeds imbibed for two days at 15°C, 17.5°C, 23°C and 15°C/23°C in darkness before the Rp. A FRp pulse control treatment is shown (seeds imbibed in darkness without a light pulse germinated less than 2%). (c) R-

mediated seed germination of Col-0, *phyA*, *phyB* and *phyA phyB* mutant seeds imbibed for two days at the different pre-incubation temperatures indicated in (a). Each bar represents mean ± SE (n≥6). Significant differences between means are shown with different letters ($p$<0.05 by ANOVA followed by Bonferroni post test).

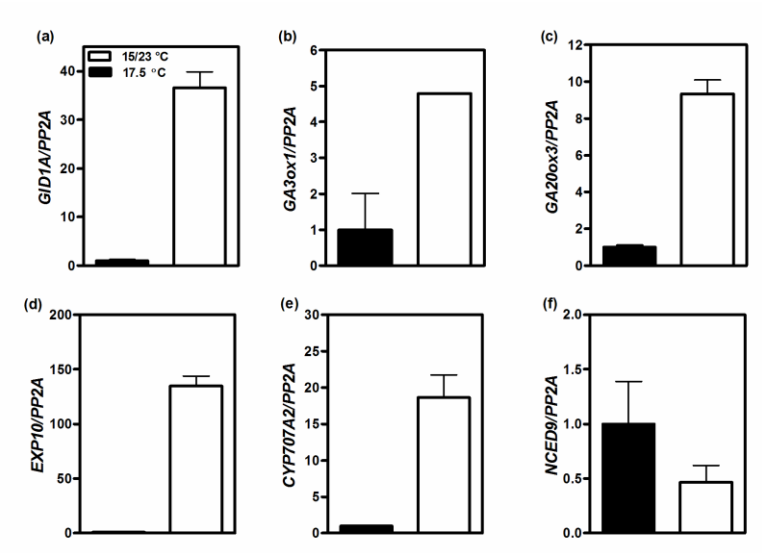


**Figure 2. Daily temperature cycles promote the expression of genes involved in ABA and GA metabolism.** (a-c) Three GA-related (*GID1A*, *GA3ox1* and *GA20ox3*), (d) one cell wall-related (*EXP10*), and (e-f) two ABA-related (*NCED9* and *CYP707A2*) genes are shown. Col-0 samples were harvested at ZT=0 before the Rp. Gene expression levels were normalized to the constant control temperature (17.5°C). Each bar represents mean ± SD. The experiment was repeated three times with similar results.

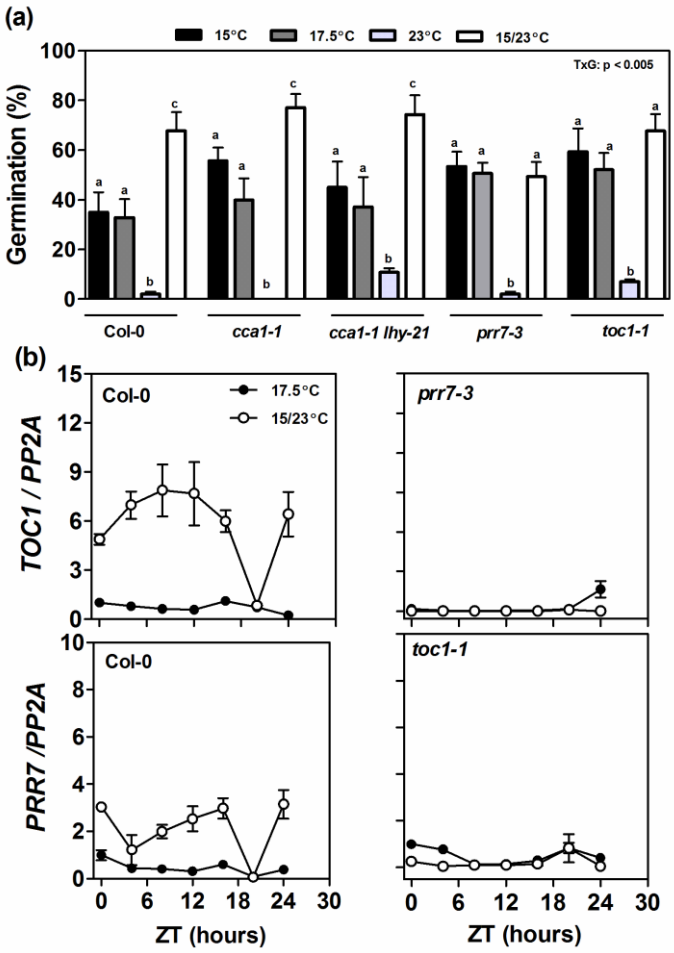


**Figure 3. *PRR7* and *TOC1* functional alleles are required for the promotion of seed germination by daily temperature cycles.** (a) R-mediated seed germination of circadian clock mutants. The experimental protocol is the same as in Figure 1a. Each bar represents mean ± SE (n=7). Significant differences between means are shown with different letters ($p$<0.05 by ANOVA followed by Bonferroni post test). (b) *TOC1* and *PRR7* expression in Col-0, *toc1-1* and *prr7-3* seeds pre-incubated at 17.5°C and 15°C/23°C. Samples were taken every four hours after the pre-incubation treatments in darkness. Each point represents mean ± SD. The experiment was repeated three times with similar results.

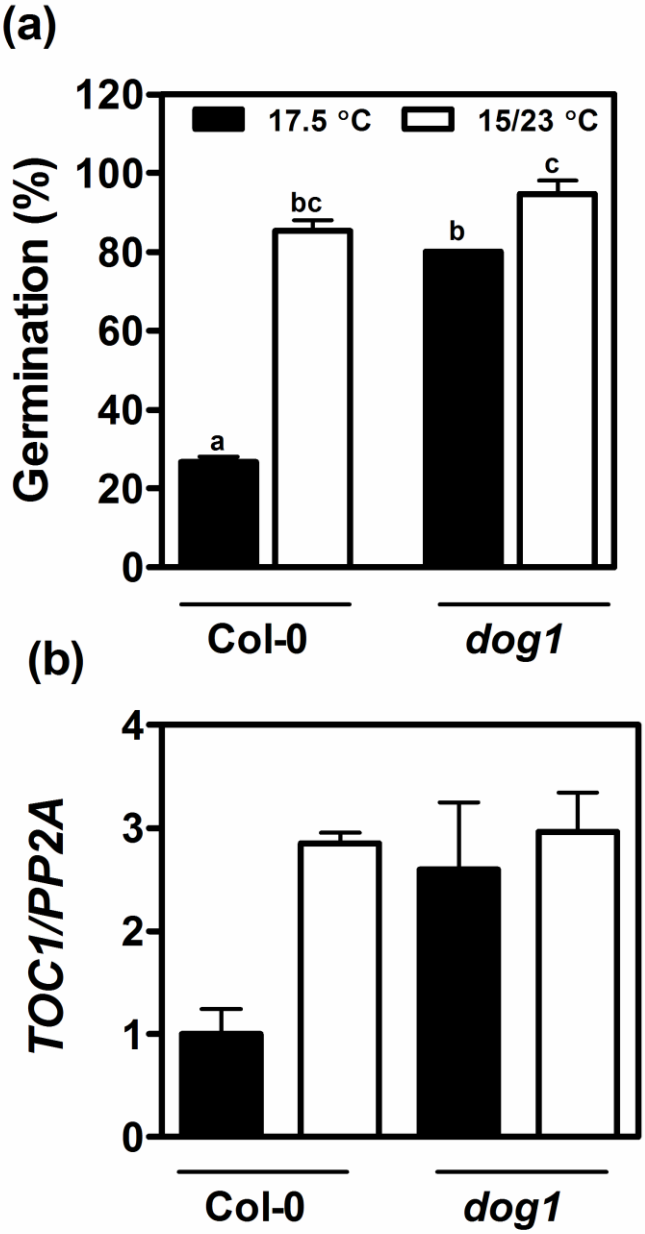


**Figure 4. DOG1 inhibits seed germination by daily temperature cycles.** (a) R-mediated seed germination of Col-0 and *dog1* seeds imbibed for two days at 17.5°C and 15°C/23°C in darkness before the Rp. Germination was counted after five days in darkness at 17.5°C. Each bar represents mean ± SE (n=3). Significant differences between means are shown with different letters ($p<0.05$ by ANOVA followed by Bonferroni post test). (b) *TOC1* expression in response to 17.5°C and 15°C/23°C in Col-0 and *dog1* seeds. Samples were

taken at ZT=0 before the Rp. Each bar represents mean ± SD. The experiment was repeated three times with similar results.

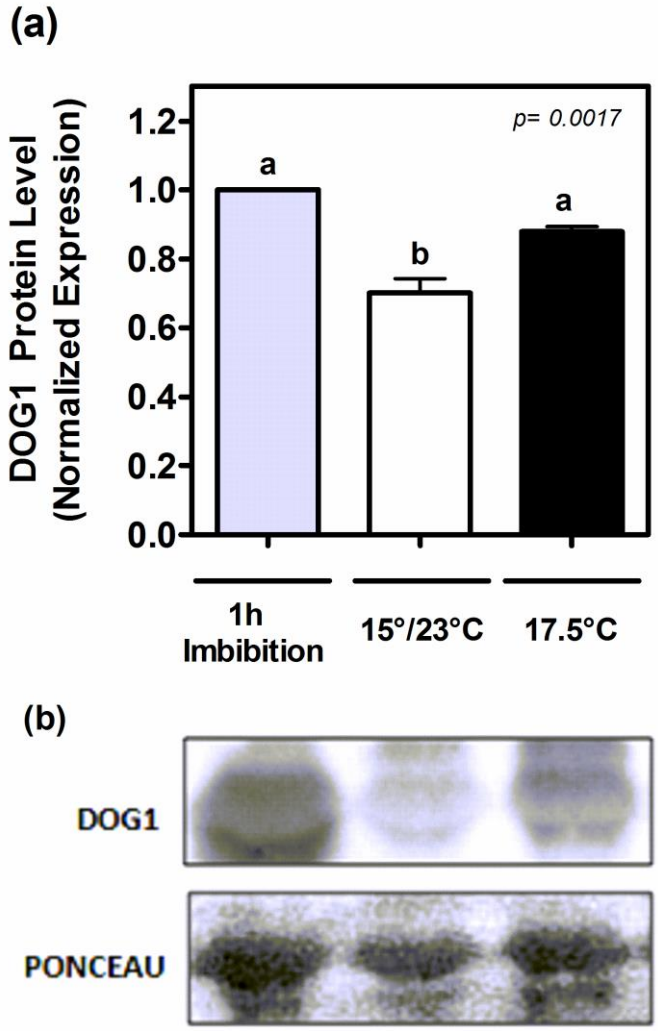


**Figure 5. Daily temperature cycles reduce DOG1 protein levels.** (a) Quantification of DOG1 protein in *ProDOG1*:*3xHA*:*DOG1* (HA-DOG1) seeds. Samples used for the western blot were taken at ZT=0, prior to the Rp. Results were normalized to the control treatment (one hour imbibition). Each bar represents mean ± SE (n=3). Significant differences between means are shown with different letters ($p$<0.05 by ANOVA followed by Bonferroni post test). (b) DOG1 protein gel corresponding to a representative experiment. DOG1 protein was detected using anti-HA antibody. Ponceau staining was used as loading control.